PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12Q 1/00, 1/60 — A1

(11) International Publicati n Number: WO 89/ 02925

(43) International Publication Date: 6 April 1989 (06.04.89)

(21) International Application Number: PCT/US88/03169

(22) International Filing Date: 20 September 1988 (20.09.88)

(31) Priority Application Number: 099,890

(32) Priority Date: 22 September 1987 (22.09.87)

(33) Priority Country: US

(60) Parent Application or Grant
(63) Related by Continuation
US 099,890 (CIP)
Filed on 22 September 1987 (22.09.87)

(71) Applicant *(for all designated States except US):* ABBOTT LABORATORIES [US/US]; D-377, Abbott Park, IL 60064 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* BATES, Diane, Marian [US/US]; 40 Meadowbrook Lane, Roanoke, TX 76262 (US). ALEJOS, Michael, A. [US/US]; 69 Evergreen Circle, Arlington, TX 76262 (US).

(74) Agents: GORMAN, Edward, Hoover, Jr.; Abbott Laboratories, D-377, AP6D-2, Abbott Park, IL 60064 (US) et al.

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: SIMULTANEOUS ASSAY FOR CHOLESTEROL AND TRIGLYCERIDES

(57) Abstract

A method for the simultaneous determination of cholesterol and triglyceride substrates with a single reagent system. The reagent system is reacted with the specimen such that each of the substrates reacts with their respective reactant simultaneously. The change in absorbance or fluorescence of the resulting reaction mixture is measured at a plurality of wavelengths which are characteristic for each of the substrates to be determined. The amount of cholesterol and triglyceride substrates can be determined by either an endpoint or reaction rate measurement. The reagent system comprises an enzyme having cholesterol esterase activity, a chromogenic oxygen acceptor, microperoxidase, and cholesterol oxidase for determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H or analogs thereof for determination of triglyceride.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**ML**	Mali
AU	Australia	**GA**	Gabon	**MR**	Mauritania
BB	Barbados	**GB**	United Kingdom	**MW**	Malawi
BE	Belgium	**HU**	Hungary	**NL**	Netherlands
BG	Bulgaria	**IT**	Italy	**NO**	Norway
BJ	Benin	**JP**	Japan	**RO**	Romania
BR	Brazil	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SN**	Senegal
CH	Switzerland	**LI**	Liechtenstein	**SU**	Soviet Union
CM	Cameroon	**LK**	Sri Lanka	**TD**	Chad
DE	Germany, Federal Republic of	**LU**	Luxembourg	**TG**	Togo
DK	Denmark	**MC**	Monaco	**US**	United States of America
FI	Finland	**MG**	Madagascar		

SIMULTANEOUS ASSAY FOR CHOLESTEROL AND TRIGLYCERIDES

BACKGROUND OF THE INVENTION

This is a continuation-in-part application of serial number 099,890, filed September 22, 1987.

The present invention relates to the simultaneous measurement of a plurality of substrates with a single reagent by monitoring concurrent reactions which produce changes in the electromagnetic radiation absorbance characteristics of the sample. In one aspect, the invention relates to the simultaneous measurement of cholesterol and triglycerides in blood serum by monitoring two concurrent reactions at two or more different wavelengths.

In the field of diagnostics, various assays are designed to identify or quantify a substrate which may be present in a sample material. Unfortunately the assay is usually only specific to one type of substrate even though it may be desirable to diagnose more than one substrate for any given sample. This leads to multiple testing on the same sample which increases diagnosis cost and decreases efficiency. It is therefore desirable to develop diagnostic testing which can identify or quantify multiple substrates in an efficient manner.

For example, cholesterol and triglycerides are two of the more common tests performed in the clinical chemistry laboratory. Analysis of cholesterol is typically done using a cholesterol esterase, peroxidase (Trinder) method (Tietz, N.W., Textbook of Clinical Chemistry, 1986, p. 883). In the Trinder method cholesterol esters are hydrolyzed by cholesterol

esterase to form free cholesterol and fatty acids. The free cholesterol is then oxidized by cholesterol oxidase which forms hydrogen peroxide. The hydrogen peroxide then reacts with peroxidase and a chromogenic oxygen acceptor to produce a color change in the 400-500 nm range.

Analysis of triglyceride is typically done using the lipase/glycerol kinase method (Tietz, N. W., Textbook of Clinical Chemistry, 1986, p. 887). Triglycerides are hydrolyzed by microbial lipase to produce glycerol and free fatty acids. Glycerol and ATP in the presence of glycerol kinase form glycerol-3-phosphate + ADP. The ADP from this reaction together with phosphoenolpyruvate react with pyruvate to form ATP and pyruvate. The pyruvate produced reacts with lactate dehydrogenase to produce lactate, with the concomitant oxidation of NADH producing a decrease in absorbance at 340 nm.

The assays mentioned above are performed with separate reagents in separate cuvettes. This costs the clinical chemistry lab time and money. By combining the two tests into one test the lab would be able to realize an increase in productivity and also a cost savings.

Combining the two tests is not a straightforward task. Conditions must be selected that allow precise measurement of both substrates. For example, combination of the traditional cholesterol oxidase and triglyceride lipase/glycerol kinase methods is eliminated by the fact that peroxidase in the cholesterol reaction would oxidize the NADH in the triglyceride reaction.

One way of combining the two assays in a single reaction vessel is to do a sequential assay (U.S. Patent 4,425,427 to Lud rer and EP Patent 133064 to Cam et al.). In a sequential assay, reagent for the first assay is added to the vessel and the reaction proceeds. At some later time a concentration is determined for the first component. Then a second reagent, which either quenches the first reaction or is added after the first reaction is complete, is added to the vessel to trigger a reaction with the second component. At some later time the concentration of the second component is determined. These reactions can either be monitored at the same wavelength or at different wavelengths (either through the use of filter wheels or diode arrays).

U.S. Patent 3,925,162 describes the simultaneous measurement of enzyme activity in body fluids. In this approach the substrate for each of the enzymes to be identified are added to a reaction medium with other reagents and changes in the absorbance or fluorescence of the resulting reaction system are measured. The present invention utilizes an approach where a single reagent system is used to simultaneously identify or quantify substrate by monitoring the electromagnetic signal of the reaction mixture.

SUMMARY OF THE INVENTION

The present invention is directed toward a method for the simultaneous determination of cholesterol and triglyceride substrates with a single reagent system in a reaction mixture. The method comprises adding a reagent system containing a reactant for each of the substrates to be determined, each reactant being

selected such that it is capable of giving a unique electromagnetic radiation absorbance for the particular substrate permits calculation of both substrate concentrations. The substrates are reacted with their respective reactant under conditions such that the reaction takes place simultaneously. The concentration of the substrates is determined by measuring changes in absorbance or fluorescence of the resulting reaction mixture at a plurality of wavelengths which are characteristic for each of the substrates to be determined.

In another aspect the present invention is a method for the simultaneous determination of cholesterol and triglyceride substrates with a single reagent system in a reaction mixture by adding a reagent system containing a chromophore for each of the substrates to be determined, each chromophore being selected such that it is capable of giving a unique absorbance band for the particular substrate and allows the determination of the other substrate. The substrates are reacted with their respective chromophore under conditions such that the reaction takes place simultaneously. The concentration of the substrates is determined by measuring changes in absorbance or fluorescence of the resulting reaction mixture at a plurality of wavelengths which are characteristic for each of the substrates to be determined.

The reagent system comprises an enzyme having cholesterol esterase activity, a chromogenic oxygen acceptor, microperoxidase, and cholesterol oxidase for determination of cholesterol; and lipase, adenosine

triphosphate (ATP), phosphoenolpyruvate (PEP), glycerol kinase, pyruvate kinase, lactate dehydrogenase (LDH) and NAD(P)H or analogs th reof for determination of triglyceride. The simultaneous assay can be performed with a reagent system comprising lipase, 4-aminoantipyrine, phenol, microperoxidase and cholesterol oxidase to allow for a reaction rate or endpoint determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH, and NAD(P)H in concentrations sufficient to allow an endpoint or reaction rate determination of triglyceride.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for simultaneously measuring a plurality of substrates in a biological fluid. The method utilizes a single reagent for measurement of each of the substrates by monitoring several electromagnetic signals simultaneously.

The electromagnetic signals can be monitored simultaneously by a spectrophotometer, or spectrofluorometer. The measurement of changes in the reaction mixture can be carried out on any of the instruments by conventional procedures. The particular change in the system ,i.e., wavelength, is not critical, but it is preferable that the changes or differences in wavelength be as great as possible provided they can be monitored simultaneously.

In a simultaneous assay a reagent containing all the components for reaction with the substrates to be measured are added to the sample and the reactions

are monitored by the instrument. Typically, a simultaneous assay is done in a single cuvette with a single reagent, eliminating the need for a second reagent dispense or other optional steps generally associated with multiple substrate assays.

A key to the design of a simultaneous assay is the selection of reagents that will allow the reactions to proceed simultaneously, and permit accurate determination of both analytes in the clinically relevant range. Reactants are chosen for each of the substrates to be determined, such that each is capable of giving a unique electromagnetic radiation absorbance for the particular substrate. A reactant can be a chromophore or indicator dye where the reaction will be monitored by spectra wavelength. For example, by choosing appropriate chromophores an assay can be developed that will measure cholesterol and triglyceride simultaneously as described below.

After the proper reactant is chosen the sample is added to the reagent system which contains the appropriate reactants. The reagent and sample are mixed such that each of the substrates is contacted with their respective reactant under conditions such that the reaction takes place simultaneously. The addition and mixing of the sample and reagent is monitored by instrumentation appropriate for the reaction taking place such as measuring changes in absorbance or fluorescence of the resulting reaction mixture at a plurality of wavelengths which are characteristic for each of the substrates to be determined.

Preferably the monitoring of the reaction mixture is begun as soon as the reagent and sample are intermixed. This allows for monitoring of changes in either the reaction rate or endpoint reaction change for the particular electromagnetic signal being monitored.

The subject method allows for the simultaneous measurement of cholesterol and triglyceride in blood serum using a single reagent. The cholesterol and triglyceride reactions proceed at the same time, with measurement of the two different reactions monitored at two separate wavelengths by a spectrophotometer. The spectrophotometer employs a diode array detector having the capability of simultaneously monitoring many wavelengths.

Generally the reagent system comprises an enzyme having cholesterol esterase activity, a chromogenic oxygen acceptor, microperoxidase, and cholesterol oxidase for the determination of cholesterol; and lipase, adenosine triphosphate (ATP), phosphoenolpyruvate (PEP), glycerol kinase, pyruvate kinase, lactate dehydrogenase (LDH) and reduced nicotinamide-adenine dinucleotide, or reduced nicotinamide-adenine dinucleotide phosphate, jointly referred to as NAD(P)H, or analogs thereof for the determination of triglyceride.

The measurement of cholesterol is through the use of microperoxidase to combine with hydrogen peroxide, and a chromogenic oxygen acceptor such as a quinoneimine dye with a cosubstrate such as phenol or dihydroxybenzoate whose absorbance range is between

400-500 nm. Preferably the chromogenic oxygen acceptor is 4-aminoantipyrine (4-AAP) and phenol to produce the final dye. This allows coupling the cholesterol reagent with a triglyceride reagent using NAD(P)H or analogs thereof, since microperoxidase does not oxidize NAD(P)H. The triglyceride reaction monitors the oxidation of NAD(P)H at 340 nm.

Another reagent system comprises an enzyme having cholesterol dehydrogenase activity and NAD(P)H or analogs thereof for the determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H or analogs thereof for the determination of triglyceride. This reagent system is an enzyme system where chromophores are chosen for cholesterol and triglycerides which have distinguishable energy spectra such that they can be simultaneously determined. Preferred chromophores for the determination of cholesterol are NAD(H), NADP(H), thio-NAD(H), thio-NADP(H), hypoxanthine-NAD(H), hypoxanthine-NADP(H), or analogs therof. Preferred chromophores for the determination of triglycerides are NAD(H), NADP(H), thio-NAD(H), thio-NADP(H), hypoxanthine-NAD(H), hypoxanthine-NADP(H), or analogs thereof. The NAD or NADP chromophores can be used in either a reduced or oxidized state as either form can be used to monitor a change in absorbance or fluorescence.

A reagent system useful in performing the above simultaneous assay comprises lipase, ATP, glycerol kinase, pyruvate kinase, NADH, phosphoenolpyruvate and lactate dehydrogenase for the determination of triglycerides, and NADP specific cholesterol dehydrogenase and thio-NADP for the determination of cholesterol.

Th absorbance maximum of thio-NADP is at 404 nm, with relatively little absorbance change at 340 nm. This allows coupling of the triglycerides reagent with the cholesterol reagent using the cholesterol dehydrogenase monitoring the reactions at the appropriate wavelengths and either endpoint or rate reactions depending upon the concentrations of the glycerol kinase and cholesterol dehydrogenase.

To further describe the instant invention the following examples are provided.

EXAMPLE 1
Cholesterol/Triglyceride Simultaneous Assay

The following procedure describes a method for performing a simultaneous assay for cholesterol and triglyceride by monitoring the endpoints of both the cholesterol and the triglyceride reactions. A reagent system was prepared by mixing the following (U/L is units per liter and mM is millimoles per liter):

Cholate,Na	3.0mM
4-Aminoantipyrine	0.8mM
Phenol	14.0mM
Lipase	250,000U/L
Cholesterol Oxidase	117U/L
Microperoxidase	12mg/L
NADH	0.4mM
Phosphoenolpyruvate (PEP)	0.7mM
Adenosine triphosphate (ATP)	0.06mM
$MgSO_4$	5.5mM
Tris buffer	100mM
Succinic Acid	26mM
Pyruvate Kinase	1667U/L
Glycerol Kinase	667U/L
Lactate Dehydrogenase (LDH)	1000U/L

Sample was added to the reagent at a ratio of 1:101 and the reaction was allowed to proceed. After 3 minutes the absorbance was read at 340 nm and at 500 nm. Concentrations were calculated by comparison with standard curves.

EXAMPLE 2

Cholesterol/Triglyceride Simultaneous Assay

The following procedure describes a method for performing a simultaneous assay for cholesterol and triglyceride by monitoring the cholesterol rate of reaction and the triglyceride reaction endpoint. A reagent system was prepared by mixing the following:

Cholate,Na	3.0mM
4-Aminoantipyrine (4-AAP)	0.8mM
Phenol	14.0mM
Lipase	250,000U/L
Cholesterol Oxidase	10U/L
Microperoxidase	12mg/L
NADH	0.4mM
Phosphoenolpyruvate (PEP)	0.7mM
ATP	0.06mM
$MgSO_4$	5.5mM
Tris buffer	100mM
Succinic Acid	26mM
Pyruvate Kinase	1667U/L
Glycerol Kinase	667U/L
Lactate Dehydrogenase (LDH)	1000U/L

Sample was added to the reagent at a ratio of 1:101 and the reaction was allowed to proceed. The cholesterol rate of reaction was monitored at 500 nm by

taking a reading every 60 seconds for three minutes, starting at 60 seconds. After 3 minutes the absorbance is read at 340 nm. Concentrations are calculated by comparison with standard curves.

EXAMPLE 3

Cholesterol/Triglyceride Simultaneous Assay

The following procedure describes a method for performing a simultaneous assay for cholesterol and triglyceride by monitoring the cholesterol reaction endpoint and the triglyceride reaction rate. A reagent system was prepared by mixing the following:

Cholate,Na	3.0mM
4-Aminoantipyrine	0.8mM
Phenol	14.0mM
Lipase	250,000U/L
Cholesterol Oxidase	117U/L
Microperoxidase	12mg/L
NADH	0.4mM
Phosphoenolpyruvate	0.7mM
ATP	0.06mM
$MgSO_4$	5.5mM
Tris buffer	100mM
Succinic Acid	26mM
Pyruvate Kinase	1667U/L
Glycerol Kinase	60U/L
LDH	1000U/L

Sample was added to the reagent at a ratio of 1:101 and the reaction was allowed to proceed. The triglyceride was followed at 340 nm by reading every 60 seconds for three minutes. After 3 minutes the absorbance is read at 500 nm. Concentrations were calculated by comparison with standard curves.

EXAMPLE 4 -12-

Cholesterol/Triglyceride Simultaneous Assay

The following procedure describes a method for performing a simultaneous assay for cholesterol and triglyceride by monitoring the cholesterol reaction rate and the triglyceride reaction rate. A reagent system was prepared by mixing the following:

Cholate,Na	3.0mM
4-Aminoantipyrine	0.8mM
Phenol	14.0mM
Lipase	250,000U/L
Cholesterol Oxidase	10U/L
Microperoxidase	12mg/L
NADH	0.4mM
Phosphoenolpyruvate	0.7mM
ATP	0.06mM
$MgSO_4$	5.5mM
Tris buffer	100mM
Succinic Acid	26mM
Pyruvate Kinase	1667U/L
Glycerol Kinase	60U/L
LDH	1000U/L

Sample was added to the reagent at a ratio of 1:101 and the reaction was allowed to proceed. Every 60 seconds for three minutes the absorbance is read at 500 nm and at 340 nm. Concentrations were calculated by comparison with standard curves.

EXAMPLE 5

Cholesterol/Triglyceride Simultaneous Fluorescent Assay

The following procedure describes a method for performing a simultaneous assay for cholesterol and triglyceride which employs fluorescence to determine the substrate concentration. In this method a spectrofluorometer is used to monitor the simultaneous reactions. The components of the assay are essentially the same as in Example 1. The cholesterol part of the assay is measured by following the fluorescence emission peak as the dye is formed. The triglyceride part of the assay is measured by following the fluorescence emission at 440 nm with excitation at 340 nm as NADH is oxidized to NAD.

EXAMPLE 6

Endpoint/Endpoint

Cholesterol Dehydrogenase	20,000U/L
thio-NADP	0.25mM
NADH	0.40mM
Phosphoenolpyruvate	0.70mM
Adenosine triphosphate	0.06mM
$MgSO_4$	5.5mM
Tris buffer	100mM
Succinic Acid	26mM
Pyruvate Kinase	1667U/L
Glycerol Kinase	667U/L
Lactate Dehydrogenase (LDH)	1000U/L

Sample is added to the reagent at a dilution ratio of 1:101 and the reaction is allowed to proceed. After 3 minutes the absorbance is read at 340 nm and 404 nm. Concentrations are calculated by comparison with standard curve.

EXAMPLE 7

Cholesterol Endpoint/Triglycerides Rate

Cholesterol Dehydrogenase	20,000U/L
thio-NADP	0.25mM
NADH	0.40mM
Phosphoenolpyruvate	0.70mM
Adenosine triphosphate	0.06mM
$MgSO_4$	5.5mM
Tris buffer	100mM
Succinic Acid	26mM
Pyruvate Kinase	1667U/L
Glycerol Kinase	60U/L
Lactate Dehydrogenase (LDH)	1000U/L

Sample is added to the reagent at a dilution ratio of 1:101 and the reaction is allowed to proceed. The triglycerides rate of reaction is monitored at 340 nm by taking a reading every 60 seconds. After 3 minutes the absorbance is read at 404 nm. Concentrations are calculated by comparison with standard curves.

EXAMPLE 8

Cholesterol Rate/Triglycerides Endpoint

Cholesterol Dehydrogenase	2000U/L
thio-NADP	0.25mM
NADH	0.40mM
Phosphoenolpyruvate	0.70mM
Adenosine triphosphate	0.06mM
$MgSO_4$	5.5mM
Tris buffer	100mM
Succinic Acid	26mM
Pyruvate Kinase	1667U/L
Glycerol Kinase	667U/L
Lactate Dehydrogenase (LDH)	1000U/L

Sample is added to the reagent at a dilution ratio of 1:101 and the reaction is allowed to proceed. The cholesterol rate of r action is monitored at 404 nm by taking a reading every 60 seconds. After 3 minutes the absorbance is read at 340 nm. Concentrations are calculated by comparison with standard curves.

EXAMPLE 9

Cholesterol Rate/Triglycerides Rate

Cholesterol Dehydrogenase	2000U/L
thio-NADP	0.25mM
NADH	0.40mM
Phosphoenolpyruvate	0.70mM
Adenosine triphosphate	0.06mM
$MgSO_4$	5.5mM
Tris buffer	100mM
Succinic Acid	26mM
Pyruvate Kinase	1667U/L
Glycerol Kinase	60U/L
Lactate Dehydrogenase (LDH)	1000U/L

Sample is added to the reagent at a dilution ratio of 1:101 and the reaction is allowed to proceed. The triglycerides rate of reaction is monitored at 340 nm by taking a reading every 60 seconds. The cholesterol rate of reaction is monitored by taking a reading every 60 seconds. Concentrations are calculated by comparison with standard curves.

WE CLAIM:

1. A method for the simultaneous determination of cholesterol and triglyceride substrates with a single reagent system in a reaction mixture, comprising:

adding a reagent system containing a reactant for each of the substrates to be determined, each reactant being selected such that it is capable of giving a unique electromagnetic radiation absorbance for the particular substrate and permits the determination of the other substrate;

simultaneously reacting each of the substrates with their respective reactant under conditions such that the reaction takes place simultaneously; and

measuring changes in absorbance or fluorescence of the resulting reaction mixture at a plurality of wavelengths which are characteristic for each of the substrates to be determined.

2. A method for the simultaneous determination of cholesterol and triglyceride substrates with a single reagent system in a reaction mixture, comprising:

adding a reagent system containing a chromophore for each of the substrates to be determined, each chromophore being selected such that it is capable of giving a unique absorbance band for the particular substrate which permits determination of the other substrate;

simultaneously reacting each of the substrates with their respective chromophore under conditions such that the reaction takes place simultaneously; and

measuring changes in absorbance or fluorescence of the resulting reaction mixture at a plurality of wavelengths which are characteristic for each of the substrates to be determined.

3. The method of Claim 2 wherein said reagent system comprises an enzyme having cholesterol esterase activity, a chromogenic oxygen acceptor, microperoxidase, and cholesterol oxidase for determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H or analogs thereof for determination of triglyceride.

4. The method of Claim 3 wherein said reagent system comprises lipase, 4-AAP, phenol, microperoxidase, cholesterol oxidase in a concentration sufficient to allow an endpoint determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H in a concentration sufficient to allow an endpoint determination of triglyceride.

5. The method of Claim 3 wherein said reagent system comprises lipase, 4-AAP, phenol, microperoxidase, cholesterol oxidase in a concentration sufficient to allow a rate determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H in a concentration sufficient to allow an endpoint determination of triglyceride.

6. The method of Claim 3 wherein said reagent system comprises lipase, 4-AAP, phenol, microperoxidase, cholesterol oxidase in a concentration sufficient to allow an endpoint determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H in a concentration sufficient to allow a rate determination of triglyceride.

7. The method of Claim 3 wher in said reagent system comprises lipase, 4-AAP, phenol, microperoxidase, cholesterol oxidas in a concentration sufficient to allow a rate determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H in a concentration sufficient to allow a rate determination of triglyceride.

8. The method of Claim 2 wherein said reagent system comprises an enzyme having cholesterol dehydrogenase activity and NAD(P)H or analogs thereof for determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H or analogs thereof for determination of triglyceride.

9. The method of Claim 9 wherein said reagent system comprises an enzyme having cholesterol dehydrogenase activity and NAD(P)H or analogs thereof in a concentration sufficient to allow an endpoint determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H or analogs thereof in a concentration sufficient to allow an endpoint determination of triglyceride.

10. The method of Claim 9 wherein said reagent system comprises an enzyme having cholesterol dehydrogenase activity and NAD(P)H or analogs thereof in a concentration sufficient to allow a rate determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H in analogs thereof in a concentration sufficient to allow an endpoint determination of triglyceride.

11. The method of claim 9 wherein said reagent system comprises an enzyme having cholesterol

dehydrogenase activity and NAD(P)H or analogs thereof in a concentration sufficient to allow an endpoint determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H or analogs thereof in a concentration sufficient to allow a rate determination of triglyceride.

12. The method of claim 9 wherein said reagent system comprises an enzyme having cholesterol dehydrogenase activity and NAD(P)H or analogs thereof in a concentration sufficient to allow a rate determination of cholesterol; and lipase, ATP, PEP, glycerol kinase, pyruvate kinase, LDH and NAD(P)H or analogs thereof in a concentration sufficient to allow a rate determination of triglyceride.

13. A reagent system for the simultaneous determination of cholesterol and triglyceride as described in Claim 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/03169

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12Q 1/00, 1/60
U.S. Cl: 435/4, 435/11

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/4, 11, 16, 18, 19, 25; 436/13, 71

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts Services Online (File CA, 1967-1988 ; File Biosis Previews 1969-1988). Automated Patent System (File USPAT, 1975-1988).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	US, A, 4,425,427 (Luderer) 10 Jaunary 1984, See Column 4, lines 4-68, and Column 5, lines 1-68.	1 2-13
Y	FR, A, 2,547,925 (Cam) 28 December 1984.	1-13
Y	US, A, 4309,502 (Lauderdale) 5 January 1982, See the Abstract.	1-13
Y	US, A, 3,925,164 (Beaucamp) 9 December 1975. See the Abstract.	8-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 November 1988	11 JAN 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Toni R. Scheiner Toni R. Scheiner

Form PCT/ISA/210 (second sheet) (Rev.11-87)

No additional fee is believed to be due. However, if any fee is made payable by the filing of this paper, please consider this our authorization to charge the deposit account of the undersigned, Deposit Account No. 06-0540.

Respectfully submitted,

10/7/02
DATE

SIGNATURE OF PRACTITIONER

Reg. No.: 36,050

R. Alan Weeks
(type or print name of practitioner)

Tel. No.: (918) 599-0621

321 S. Boston Ave., Suite 800
P.O. Address

Customer No.: 22206

Tulsa, OK 74103-3318

171026.1

22206
PATENT TRADEMARK OFFICE